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High-performance liquid chromatography of globin chains in the identification of human globin gene abnormalities

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This article summarizes experience and data obtained using a previously developed reverse-phase high-performance liquid chromatography method (J.B. Shelton, J.R. Shelton and W.A. Schroeder, J. Liq. Chromatogr. 7 (1984) 1969) in the study of a number of hemoglobinopathies in the Sardinian population. The occurrence and incidence of several abnormal hemoglobins are described, as well as aspects of the expression of abnormal γ-globin gene arrangements and thalassemic genes.

1. Introduction

In the past three decades, chromatography has played an important role in the separation and characterization of normal and abnormal hemoglobins (Hb) and globins [1]. The advent of high-performance liquid chromatography (HPLC) has further influenced and stimulated research in this field. Thanks to a great reduction in the time required to perform an analysis, the requirement of only micro-quantities of material for each determination, and the possibility of automatization, HPLC has become the preferred methodology for the study of several types of hemoglobinopathies at both screening and research level. Two major HPLC approaches are widely used: ion-exchange and reverse-phase. The former allows normal and abnormal hemoglobin tetramers to be separated [2–4], whereas the latter greatly facilitates the separation and isolation of the globin chains [5–8] and globin chain proteolytic fragments for subsequent characterization of the amino acid substitution within the abnormal peptide [6,9]. Reverse-phase HPLC also allows the amino acid composition of a chemically cleaved peptide to be determined in a few minutes at the picomole [10] or sub-nanomole level [11].

Since 1949 when the Hb S was discovered in sickle cell anemia lysates, the number of Hb variants has increased to 526 (April, 1989) [12] while about 100 different types of thalassemia and related syndromes have been characterized so far. In the past few years, the presence of a relevant number of hemoglobinopathies (other than β-thalassemia) among the population of the island of Sardinia has become evident. With the aim of searching for Hb structure abnormalities and globin synthesis defects in the population, we adopted a strategy involving two electrophoretic procedures followed by a subsequent investigation by HPLC of globin chains when an altered pattern is present. The two electrophoretic methods, which are not described in detail here, are the conventional isoelectric focusing of the native tetramers [13–15], and the separation of constituent globin chains by the dissociating gel electrophoresis de-

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veloped by Alter et al. [16]. Application of these procedures to blood samples during population studies allows the identification of variants with an isoelectric point differing from that of normal Hbs by 0.02 pH units, as well as variants exhibiting a silent amino acid substitution which simply alters the hydrophobicity of the globin chain [15,17–20]. The same strategy has been successfully adopted by Lacombe et al. [21] for the identification of known silent β-chain variants in order to avoid unnecessary protein structure determination. Moreover, the association of this strategy with studies at genomic level allowed us to understand more clearly the expression of several abnormal globin gene arrangements in adult and newborns [22–30]. The separation of globin chains by HPLC and its relevance in this study will be discussed here.

2. Materials and methods

Blood samples were collected and treated following standard procedures routinely in use in our laboratory as recommended and described by Wilson and Huisman [2]. Globin chain separation followed the reverse-phase HPLC described by Shelton et al. [5,6] with minor modifications. This method is also widely used, with slight modifications, in the laboratory of T.H.J. Huisman where most of the work in this field has been carried out [2–4,7–8] as well as in other laboratories. Basically, lysates containing 0.05–0.1 mg of Hb in 20–50 μl of mixture A (see below) were filtered through HV filters (0.45 μm; Millipore, Bedford, MA) and injected in the Vydac large-pore (300 Å) C_4 column (4.6 × 250 mm, catalog no. 214TP54, from the Separations Group, Hesperia, CA). The column was previously equilibrated with a fixed volume of 20 ml of 50% mixture A + B. The chromatogram was developed with a linear gradient between mixture A (20% acetonitrile–80% water containing 0.1% trifluoroacetic acid (TFA)) and mixture B (60% acetonitrile–40% water containing 0.1% TFA). The most suitable gradient, especially in the case of mixtures containing fetal globin chains, was 50–60% mixture B in 80 min at a flow rate of 1 ml/min at room temperature. Absorbance was monitored at 220 nm, and recording was at 0.05 absorbance units full scale with a chart speed of 12 cm/h. A 5 min gradient was run to return to the 50% mixture B, and equilibration was effected with 20 ml before the next chromatogram was started. Purging was performed after five runs by a 5 min gradient to 100% mixture B which was followed by an isocratic development with 100% mixture B for 15 min. A 5 min gradient was finally applied to return to the 50% mixture B and equilibration was continued until a 20 min constant absorbance was achieved. Reagents were HPLC grade, filtration was unnecessary, and mixtures were sonicated soon after preparation. Mixtures were degassed each working day with a low stream of helium for 1–2 min. No significant differences in retention times were observed when using the same column in two different HPLC equipments.

3. Results and discussion

Under the above-described conditions of development, the elution of the normal chains is highly reproducible although retention times may depend on the composition of the developers, the condition of the column and the number of performed runs, the temperature, etc. Because of the presence of 0.1% TFA, a pH of 2.2–2.5 is reached in the solvent which advantageously dissociates Hb into heme and the constituent globin chains directly into the column. Small changes in pH have a minimal effect on the separation. Since the chromatographic behavior of globins in this system is sensitive to the condition of the development [5], small modifications of the slope of the gradient might considerably improve the separation of a variant peptide. The hydrophobicity of the polypeptide greatly affects the separation of the globin chains. Huisman [3,4] suggested a ranking of the 20 amino acids on the basis of polarity, size and pK values at pH < 3 (as is the case in the method described here), by which means it is possible to predict a modification in retention time due to the exchange of the amino acid residue. The sequence is as follows: Trp, Phe, Ile, Leu, Tyr, Pro, Met, Val, Thr, Ala, Gly, Asp, Glu, Asn, Cys, Gln, Ser,

His, Lys, Arg. The substitution of a residue by one listed to its right (being more hydrophilic) will decrease the elution time of the globin chain, and vice versa. The author tested this useful principle with 46 different variants [4]. In all cases, the observed changes in mobility agreed with those predicted. Variants shown in this report, plus 22 more described in the literature of the past three years, support the validity of the principle and indicate that the proposed list is a helpful guide in search for substitution in a globin chain which is readily separating from its normal counterpart. Some exceptions have been found in evaluating data for variants in which a glycine residue is involved [3]. Similarly, comparison of seven variants with the substitution Glu → Lys, three with the Pro residue replaced by Thr, Ser, or Arg, and one with an Ala replaced by Pro [3,31–33], where the more polar properties of the replacing residues would be expected to result in faster elution than that observed, clearly indicated that the location of the substitution within the polypeptide could have a considerable effect on the retention time.

Fig. 1 is an example of the separation of normal globin chains and indicates the position of

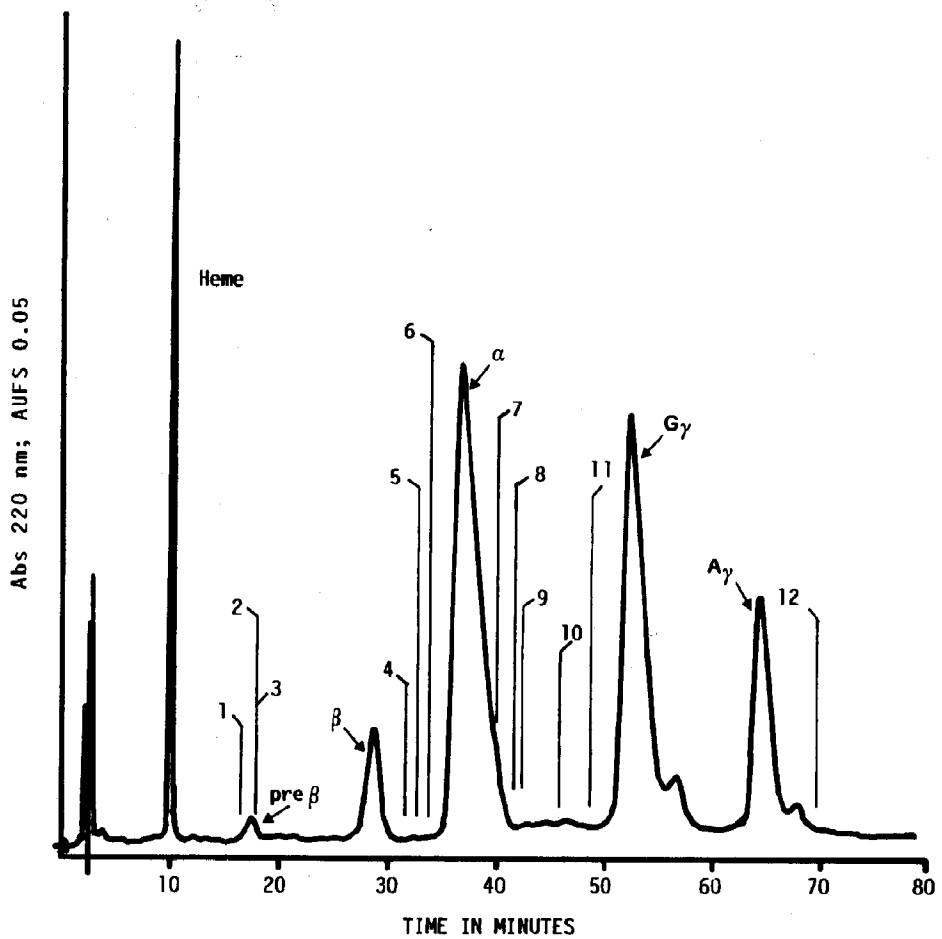

Fig. 1. HPL chromatogram of globins of a normal newborn showing retention times of Hb variants found in the Sardinian population. (1) β-C (6Glu → Lys), (2) δβ-Lepore-Baltimore, (3) α-Sassari (126Asp → His); (4) β-Hamilton (11Val → Ile), (5) α-G-Philadelphia (68Asn → Lys), (6) α-O type; (7) α-J type, (8) α-J-Sardegna (50His → Asp), (9) $^{A}\gamma$ unidentified, (10) $^{A}\gamma^{T}$ (75Ile → Thr), (11) $^{G}\gamma$-Malta (117His → Arg), (12) $^{A}\gamma$ unidentified.

variants we found among Sardinians. Many other variants are at present under investigation. It is interesting that most of the mutants occur with a surprisingly high prevalence, exceptions being some recently observed variants, such as Hb Lepore-Baltimore [20], and others of uncertain origin. The Hb J-Sardegna (α50His → Asp) has a 0.25% incidence in the northern part of the island [19]. This incidence is probably the highest so far found in humans of a variant not associated with an α-thalassemia gene in *cis*, which would have had a selective advantage in a malarial area. Hb G-Philadelphia (α68Asn → Lys), which by contrast may be linked to an α-thalassemia gene, also has a high 1:1500–1:1800 incidence [15]. This value appears to be higher than the incidence found in Black populations where the variant is assumed to have originated. The incidence of the same Hbs in the southern area of the island is 10-fold lower. The silent Hb Hamilton (β11Val → Ile) shows a considerably high incidence (0.15–0.20%) [17], but the reason for this is only speculative. This variant does not produce any functional alterations and is probably not restricted to Sardinians. Particular attention was focussed upon the silent Hb F-Sardinia ($^{A}\gamma$75Ile → Thr) in our laboratory. It has the high 0.35% incidence which is among the highest so far described among populations with different ethnic backgrounds [14,22,27,30]. The Hb F-Malta I ($^{G}\gamma$117His → Arg) occurs only in newborns in a village situated near the city of Sassari. Its incidence is about 1:2500. It is at present not possible to speculate about the origin of this mutant which was considered restricted to the Maltese population where it attains a 1.5% frequency [34]. Its relatively high frequency, and the possibility of completely separating the abnormal chain by HPLC, should facilitate future investigations on the reason for lower than expected percentages of this variant at birth. The Hb Sassari (α126Asp → His) [18] has been found in three apparently unrelated families of a single little town. All these data may be evidence of different founder effects and of a quite high inbreeding of the Sardinian population.

The reverse-phase HPLC on the C_4 column greatly facilitated quantitation of the $^{G}\gamma$ and $^{A}\gamma$ chains of Hb F and, which is of importance, the frequently observed $^{A}\gamma^{T}$ variant which has an Ile → Thr substitution at position 75 of the $^{A}\gamma$ chain [35]. This variant is also known as the Hb F-Sardinia; it has a high frequency in many populations [30,36], and its detection and quantitation may be of great importance in determining the expression of a particular globin gene being located *cis* or *trans* to it. Association of DNA technology with HPLC of globins has provided data confirming previous observations, and has extended information about abnormal γ-globin gene arrangements and expression. A study of as many as 8000 Sardinian newborns by means of globin gene mapping and fetal Hb analysis has identified five different arrangements (all of them in heterozygosity with the normal $-^{G}\gamma-^{A}\gamma-$ or with the $-^{G}\gamma-^{A}\gamma^{T}-$ arrangements). These arrangements were determined by (at least) eight different unequal crossing-over events: (A) Two types of the $-^{GA}\gamma-$ and one type of the $-^{GA}\gamma^{T}-$ hybrid thalassemic gene directing the synthesis of the $^{A}\gamma$ (or the $^{A}\gamma^{T}$) chain, thus resulting in a 40% $^{G}\gamma$ chain at birth instead of the normal 70%, and decreased Hb F level; (B) The $-^{A}\gamma-^{A}\gamma^{T}-$ duplication characterized by the lower 36% $^{G}\gamma$ level; (C) The $-^{G}\gamma-^{G}\gamma-$ duplication (88% of the $^{G}\gamma$ chain); (D) Two types of the $-^{G}\gamma-^{AG}\gamma-^{A}\gamma-$ triplication (85% $^{G}\gamma$); (E) The $-^{G}\gamma-^{G}\gamma-^{A}\gamma-$ triplication (83% $^{G}\gamma$). The same arrangements are present with quite different incidences among different ethnic groups, in which quadruplications and quintuplications also occur (see ref. 37 for a review). Table 1 compares the percentages of $^{G}\gamma$ globin, and the level of Hb A, in newborn babies with the above abnormal γ-

Table 1

Globin chain synthesis in newborn carrier of abnormal γ-globin gene arrangements

Normal values: $^{G}\gamma$, 69.7±3.0%; Hb A, 20.0±5.0%.

Arrangement	% $^{G}\gamma$-chain	% Hb A	% of total abnormal arrangements
$-^{GA}\gamma-$	38.3±5.4	34.7±4.6	21.6
$-^{A}\gamma^{I}-^{A}\gamma^{T}-$	35.6±3.4	14.1±5.9	10
$-^{G}\gamma-^{AG}\gamma-^{A}\gamma-$	84.8±2.6	9.8±4.4	21.6
$-^{G}\gamma-^{G}\gamma-^{A}\gamma-$	82.6	22.2	45
$-^{G}\gamma-^{G}\gamma-$	87.4±3.1	19.7±6.7	1.7

globin gene arrangements. It is likely that the higher $^{G}\gamma$ level in carriers of the hybrid $-^{GA}\gamma-$ gene (which produces a globin of the $^{A}\gamma$ type), compared to the corresponding level in carriers of the $-^{A}\gamma-^{A}\gamma^{T}-$ duplication, depends on the presence of the more active $^{G}\gamma$ promoter in its 5′ block. The lower $^{G}\gamma$ level in the $-^{A}\gamma-^{A}\gamma^{T}-$ duplication could reflect the activity of the remaining $^{G}\gamma$ gene in *trans*, which is about 35%. Similarly, the $^{G}\gamma$ level of the triplicated $-^{G}\gamma-^{AG}\gamma-^{A}\gamma-$ arrangement is lower than that of the $-^{G}\gamma-^{G}\gamma-$ because of the presence of the $^{A}\gamma$ promoter in the inserted $-^{AG}\gamma-$ hybrid gene. Also of interest is the finding that the activity of the $^{A}\gamma$ gene in the 3′ position of the triplicated and quadruplicated arrangements is appreciably decreased (60–80% lower) compared to the corresponding value in the normal $-^{G}\gamma-^{A}\gamma-$ arrangement [27,38].

Reversed-phase HPLC has been the most useful technique in studies on the heterogeneity of Hb F in adults, although the low level of this protein often requires isolation with the use of complex methods; moreover, evaluation is not very accurate. Notwithstanding, data could be collected supporting evidence on the effect of a base change at position −158 of the $^{G}\gamma$ gene in increasing Hb F and $^{G}\gamma$ globin levels in normal adult [30]. Protein studies were simple in hematologically normal adults with Hb F levels from 9 to 30% having specific forms of nondeletional hereditary persistence of Hb F (HPFH). Two types of the three nondeletional HPFH occurring on this island [39–41] have been studied in our laboratory: the Sardinian type of $^{A}\gamma$-HPFH which is due to the G to A transition at position −117 of the $^{A}\gamma$ gene, and the $^{G}\gamma$-HPFH which is due to the T to C transition at position −175 of the $^{G}\gamma$ gene. In the $^{A}\gamma$-HPFH a 11.8–16.4% Hb F synthesis was shown containing 91.5 to about 100% $^{A}\gamma$ chains [28]; in the $^{G}\gamma$-HPFH a 33% Hb F synthesis, almost completely of the $^{G}\gamma$ type, was observed (unpublished result).

We have extensively studied Hb F in homozygous β-thalassemia and correlated protein composition with haplotypes at the β-globin gene cluster and type of mutation. Results suggest a correlation between the haplotype and β-globin gene arrangement and expression: only haplotypes II and VI are associated with the $^{A}\gamma^{T}$ gene, all others being associated with the $^{A}\gamma^{I}$ gene; $^{G}\gamma$ chain production in adult β-thalassemia patient is lower in haplotype I/I with respect to haplotype II/II (50–56% vs 58–61%), $^{G}\gamma$ level in haplotype I/II is intermediate (54–55%); the synthesis of the $^{A}\gamma^{T}$ globin is significantly lower than that of the $^{A}\gamma^{I}$ globin. These data seem to indicate that a 4 bp deletion previously described in Blacks by Gilman et al. [42] as being localized in the promoter region (at −222 to −225) of the $^{A}\gamma$ gene and as having the effect of reducing the $^{A}\gamma$ gene expression in adults, affects only the promoter of the $^{A}\gamma^{T}$ gene [29]. Moreover, HPLC separation of globin chains of a baby who was a carrier of the Hb Lepore-Baltimore gene and of the $^{A}\gamma^{T}$ gene in the normal chromosome, showed a 40% lower level of the $^{A}\gamma^{T}$ globin compared to the $^{A}\gamma^{I}$ [20], clearly indicating that the 4 bp deletion might be present in the promoter of the $^{A}\gamma^{T}$ gene also in the absence of the β-thalassemia gene in *cis*. Work is in progress to investigate more extensively γ-chain composition in normal newborns in order to verify the presence of that deletion in nonthalassemic chromosomes.

It may be concluded that, apart from the biochemical and physiological importance of studies on new variant Hbs, careful and continued determination of the Hb F composition in (apparently) normal individuals and in syndromes characterized by elevated Hb F levels, will continue to extend knowledge about hemoglobin synthesis defects and will contribute to the identification of DNA segments as being part of the controlling mechanisms of globin genes, and to further knowledge of how mutations influence globin gene expression. HPLC will probably continue to be most useful in this field of analysis.

References

1 W.A. Schroeder and T.H.J. Huisman, The chromatography of hemoglobin (Dekker, New York, 1980).
2 J.B. Wilson and T.H.J. Huisman, in: The hemoglobinopathies, ed. T.H.J. Huisman (Churchill Livingstone, Edinburgh, 1986) p. 32.
3 T.H.J. Huisman, J. Chromatogr. 418 (1987) 277.
4 T.H.J. Huisman, Acta Haematol. 78 (1987) 123.

5 J.B. Shelton, J.R. Shelton and W.A. Schroeder, J. Liq. Chromatogr. 7 (1984) 1969.
6 W.A. Schroeder, in: The hemoglobinopathies, ed. T.H.J. Huisman (Churchill Livingstone, Edinburgh, 1986) p. 143.
7 F. Kutlar, A. Kutlar and T.H.J. Huisman, J. Chromatogr. 357 (1986) 147.
8 F. Kutlar, Y.J. Fei, J.B. Wilson, A. Kutlar and T.H.J. Huisman, J. Chromatogr. 394 (1987) 333.
9 J.B. Wilson, H. Lam, P. Pravatmuang and T.H.J. Huisman, J. Chromatogr. 179 (1979) 271.
10 B.A. Bidlingmeyer, S.A. Cohen and T.L. Tarvin, J. Chromatogr. 336 (1984) 93.
11 V. Baudin and H. Wajcman, Clin. Chim. Acta 162 (1987) 162.
12 International Hemoglobin Information Center Variant List, Hemoglobin 13 (1989) 221.
13 G. Cossu, M. Manca, M.G. Pirastru, R. Bullitta, A. Bianchi Bosisio, E. Gianazza and P.G. Righetti, Am. J. Hematol. 13 (1982) 149.
14 B. Masala, L. Manca, M. Formato and A. Matera, Am. J. Hematol. 21 (1986) 367.
15 L. Manca, P. Demuro and B. Masala, Clin. Chim. Acta 177 (1988) 231.
16 B.P. Alter, S.C. Goff, D.G. Efremov, M.E. Gravely and T.H.J. Huisman, Br. J. Haematol. 44 (1980) 527.
17 L. Manca, M. Formato, B. Masala, D. Gallisai and M. Orzalesi, Hemoglobin 11 (1987) 161.
18 B. Masala, L. Manca, A. Stangoni, G.B. Cuccuru, J.B. Wilson, B.B. Webber, A. Kutlar and T.H.J. Huisman, Hemoglobin 11 (1987) 373.
19 L. Manca and B. Masala, Hemoglobin 13 (1989) 33.
20 B. Masala, L. Manca, J.B. Wilson, B.B. Webber, A. Kutlar and T.H.J. Huisman, Hemoglobin 14 (1990) 241.
21 C. Lacombe, J. Riou, C. Godard, J. Rosa and F. Galacteros, Acta Haematol. 78 (1987) 119.
22 L. Manca, M. Formato, P. Demuro, G. Pilo, D. Gallisai, M. Orzalesi and B. Masala, Hemoglobin 10 (1986) 519.
23 B. Masala, M. Formato, L. Manca, P. Demuro, D. Gallisai, F. Dore and M. Longinotti, Acta Haematol. 76 (1986) 208.
24 Y. Hattori, F. Kutlar, S.S. Chen, T.H.J. Huisman, P. Demuro, M. Formato, L. Manca and B. Masala, Biochem. Genet. 24 (1986) 669.
25 B. Masala, L. Manca, D. Gallisai, A. Stangoni, K.D. Lanclos, F. Kutlar, K.G. Yang and T.H.J. Huisman, Hemoglobin 12 (1988) 661.
26 L. Oggiano, F. Dore, P. Pistidda, L. Guiso, L. Manca, B. Masala, M. Pirastu, C. Rosatelli, A. Cao and M. Longinotti, Hemoglobin 12 (1988) 673.
27 L. Manca, B. Masala, M. Orzalesi, H.J. Huang and T.H.J. Huisman, Hemoglobin 12 (1988) 741.
28 K.G. Yang, T.A. Stoming, Y.J. Fei, S. Liang, S.C. Wong, B. Masala, R.B. Huang, Z.P. Wei and T.H.J. Huisman, Blood 71 (1988) 1414.
29 L. Manca, D. Gallisai, B. Masala and J.G. Gilman, Blood 72 (1988) 66a.
30 Y.J. Fei, F. Kutlar, H.F. Harris, M.M. Wilson, A. Milana, P. Sciacca, G. Schilirò, B. Masala, L. Manca, C. Altay, A. Gurgey, J.M. de Pablos, A. Villegas and T.H.J. Huisman, Hemoglobin 13 (1989) 45.
31 A. Mrad, Y. Blouquit, C. Lacombe, R. Blibech, N. Arous, J. Bardakdjian, R. Kastally, J. Rosa and F. Galacteros, Hemoglobin 12 (1988) 23.
32 M.A.M. Ali, P. Pinkerton, S.W.S. Chow, S.D. Zaetz, J.B. Wilson, B.B. Webber, H. Hu, A. Kutlar and T.H.J. Huisman, Hemoglobin 12 (1988) 137.
33 L. Ulukutlu, H. Ozsahin, J.B. Wilson, B.B. Webber, H. Hu, A. Kutlar, F. Kutlar and T.H.J. Huisman, Hemoglobin 13 (1989) 509.
34 M.N. Cauchi, J.B. Clegg and D.J. Weatherall, Nature 223 (1969) 311.
35 V. Grifoni, H. Kamuzora, H. Lehmann and D. Charlesworth, Acta Haematol. 53 (1975) 347.
36 T.H.J. Huisman, F. Kutlar, T. Nakatsuji, A. Bruce-Tagoe, Y. Kilinc, M.N. Cauchi and C. Romero Garcia, Hum. Genet. 71 (1985) 127.
37 T.H.J. Huisman, Acta Haematol. 78 (1987) 80.
38 K. Harano, T. Harano, F. Kutlar and T.H.J. Huisman, FEBS Lett. 190 (1985) 45.
39 M. Pirastu, Y.W. Kan, R. Galanello and A. Cao, Science 223 (1984) 929.
40 S. Ottolenghi, C. Camaschella, P. Comi, B. Giglioni, M. Longinotti, L. Oggiano, F. Dore, G. Sciarratta, G. Ivaldi, G. Saglio, A. Serra, A. Loi and M. Pirastu, Hum. Genet. 79 (1988) 13.
41 S. Ottolenghi, S. Nicolis, R. Taramelli, N. Malgaretti, R. Mantovani, P. Comi, B. Giglioni, M. Longinotti, F. Dore, L. Oggiano, P. Pistidda, A. Serra, C. Camaschella and G. Saglio, Blood 71 (1988) 815.
42 J.G. Gilman, M.E. Johnson and N. Mishima, Br. J. Haematol. 68 (1988) 455.